

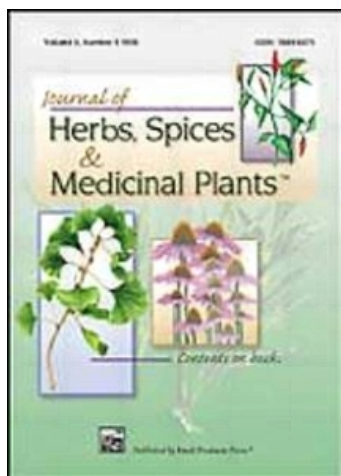
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# Screening of Nine *Echinacea* Supplements for Antitumor Activity Using the Potato Disc Bioassay

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**ABSTRACT.** To study the efficacy of commercially available *Echinacea* supplements, solvent fractions from nine locally purchased supplements containing *Echinacea* were tested in a potato disc assay for their ability to suppress formation of crown-gall tumors, a process that resembles tumor formation in animal tissues. Acetone and ethanol fractions from two supplements inhibited tumor formation and water and ethanol fractions from a third supplement suppressed tumor formation. Comparison of the bioassay results with the supplement ingredients, as listed on the supplement label, did not reveal any correlation between quantity and suppressive activity of the listed ingredients. These results are consistent with prior investigations that noted product labels were often inaccurate and that post-harvest handling practices can be deleterious to bioactive compounds contained in *Echinacea*. doi:10.1300/J044v12n01\_10 [Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <<http://www.HaworthPress.com>> © 2006 by The Haworth Press, Inc. All rights reserved.]

**KEYWORDS.** Bioactivity, dietary supplement, efficacy, herbal supplement, medicinal plant

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## INTRODUCTION

Increased interest in medicinal herbs has resulted in world retail trade valued at about \$US 12 billion in 1994, with 50 percent accounted for in Europe and 12 percent in North America (29). *Echinacea* species ranked highest in sales and accounted for almost 10 percent of market share in the United States (26). Although marketed *Echinacea* products are generally a combination of roots and aerial parts of plants (9), bioactive compounds also occur in seeds (4). Since the concentration and variety of potentially medicinal compounds vary within plants, commercial products derived from different plant organs may differ in activity and efficacy. Furthermore, the probability that different preparations of *Echinacea* (tablets, capsules, ethanol preparations, spray-dried powders, and expressed juice) would have different pharmacological effects in the human body could be expected (29). Therefore, quality control of marketed products through standardization is necessary. Consumers and regulatory authorities are becoming more concerned that retail medicinal herb formulations contain adequate levels of active constituents to ensure consistent medical efficacy (29).

Techniques have been developed to separate, identify, and quantify compounds in *Echinacea* species of potential pharmacological value (8,24). Bioactive compounds include caffeic acid phenols, polysaccharides, and unsaturated lipophilic components (29). Such constituents contribute to the immunostimulatory and antitumor potential reported for *Echinacea* (9,20). The antitumor activity of *Echinacea* is believed to be mediated by stimulation of the host's immune system, as suggested for other plants (14).

Several bioassays have been developed for the screening of active molecules of medicinal plants. These include the brine shrimp (*Artemia salina* Leach) (18,19), the lettuce (*Lactuca sativa*) seedling growth (27,32), and the potato disc (1,21) assays. Crown-gall is a neoplastic disease of plants caused by *Agrobacterium tumefaciens* and characterized by the transformation of normal plant cells into autonomous tumor cells. Previous studies have shown similarities between crown-gall tumors and animal cancer, especially the correlation between antileukemic activity and the inhibition of crown-gall tumor formation on potato discs by some medicinal herbs (2,15).

The objective of this study was to extract commercially available *Echinacea* products for antitumor activity using the potato disc bioassay.

## MATERIALS AND METHODS

**Plant material.** Herbal supplements of *Echinacea*, purchased at local grocery stores (Brands 2, 4-7) and drugstores (Brands 1, 3, 8, 9) in Knoxville, TN, were used in this study (Table 1). Random samples of each supplement, consisting of the maximum dose suggested on the container label, were used as experimental material. Tablets and capsules were ground by mortar and pestle and then extracted three times with 70 percent ethanol, acetone, or deionized water while under ultrasonic (Branson 8200 sonicator, Danbury, CT) treatments for 5 min (8). The sample to total solvent volume ratio was two percent (w/v). Successive aliquots of each drug sample extract were pooled and evaporated at 40°C under air. Dried residues were resuspended in 5 ml of a 12.5 per-

TABLE 1. Labeled contents of herbal supplements tested for antitumor activity.

Supplement	Maximum daily dose (mg)	Single unit dose (mg)	Ingredients <sup>1</sup>
Brand 1	45	15	Roots of <i>E. angustifolia</i>
	105	35	Aerial parts of <i>E. angustifolia</i>
	150	50	Aerial parts of <i>E. purpurea</i>
	1500	500	Vitamin C
Brand 2	250	125	Extract of roots of <i>E. purpurea</i> & <i>E. angustifolia</i> , containing 4 percent phenolic compounds
Brand 3	100	100	Extract of aerial parts of <i>E. purpurea</i> , containing 4 percent phenolic compounds
Brand 4	2280	380	Aerial parts of <i>E. purpurea</i>
Brand 5	1140	380	Aerial parts of <i>E. purpurea</i>
Brand 6	100	25	Extract of aerial parts of <i>E. purpurea</i> , containing 4 percent phenolic compounds
	1500	375	Aerial parts of <i>E. purpurea</i>
Brand 7	1600	400	Aerial parts of <i>E. purpurea</i>
Brand 8	750	125	Extract of leaves of <i>E. purpurea</i> , containing 4 percent phenolic compounds
Brand 9	45	15	Roots of <i>E. angustifolia</i>
	105	35	Aerial parts of <i>E. angustifolia</i>
	150	50	Aerial parts of <i>E. purpurea</i>
	1500	500	Vitamin C

<sup>1</sup> The ingredients as listed for each brand were present at all doses.

cent dimethyl sulfoxide (DMSO) solution (13) and filtered through 0.2 mm Nalgene nylon filters for sterilization.

**Bioassay.** The potato disc bioassay for antitumor activity used the bacterium *Agrobacterium tumefaciens* strain C58 that was provided by Dr. B. Ownley, Department of Entomology and Plant Pathology, University of Tennessee. The bacterium was grown in 0.8 percent nutrient broth (Becton Dickinson, Cockeysville, MD) supplemented with 0.5 percent sucrose (Fisher, Fair Lawn, NJ) and 0.1 percent yeast extract (Difco, Sparks, MD). The supplemented medium was solidified with 1.5 percent agar (Difco, Sparks, MD) as required.

The potato discs used in the assay, made from medium size tubers of "Russett" potato (*Solanum tuberosum* L.) purchased from a local supermarket and stored at 12°C until needed, were prepared according to procedures outlined by Ferrigni et al. (13). The potato tubers were surface sterilized with a 20 percent sodium hypochlorite solution for 20 min, both ends of each tuber were cut to remove and discard terminal tips, and the tubers minus ends were soaked for an additional 10 min in a second 20 percent sodium hypochlorite solution. A cork borer was then used to remove two cylinders of 2 cm in diameter from each tuber. From the cylinders, 0.5 cm-thick discs were sliced (a tuber could yield 15 discs) and one disc from each of five potatoes (five discs total) was placed on agar plates (15 g agar dissolved in 985 ml water and autoclaved for 20 min at 121°C). A potato tuber disc from each of five potatoes were placed (5 discs per plate) were inoculated within 1 h after disc preparation.

For testing the antitumor activity of each extract, a single colony of *A. tumefaciens* growing on the supplemented medium agar plate was used to loop-inoculate 50 ml of supplemented nutrient broth, which was subsequently incubated at 25°C for 48 h on a rotary shaker at 170 rpm (preliminary tests indicated that a 48 h-culture contained  $5-7 \times 10^9$  CFU/ml). After the incubation period, the bacterial suspension was serially diluted and 100 ml of each dilution was plated on supplemented nutrient agar (exact bacterial counts were determined after 48 h at 28°C). Filter-sterilized extract was diluted with sterile, deionized water (1 ml extract with 3 ml water) and then one ml of the diluted extract was combined aseptically with 1 ml of the bacterial suspension. After mixing, 100 µl of the combined extract and bacterial suspension were used to inoculate the potato discs.

After 2 days in the dark at 28°C, the plates containing the inoculated discs were wrapped with Parafilm and placed in the dark at 28°C for 18 more days. At 20 days after inoculation, the tumors on the potato discs stained with Lugol's solution and counted.

A bacterial growth inhibition test of each fraction, using a paper filter technique, was also done. Aliquots of a bacterial suspension containing  $5$  to  $7 \times 10^6$  CFU/ml were spread on a supplemented nutrient broth agar plate and a disc (4 mm in diameter) cut from Whatman No. 1 filter paper was placed on the agar surface in the plate and 10  $\mu$ l of each extract was added to the paper disc. After incubation at 28°C for 48 h, growth inhibition of *A. tumefaciens* by the fractions was detected and measured.

Experimental controls, used in tests in the same manner as the extracts, were a DMSO control (0.5 ml DMSO combined with 1.5 ml sterile deionized water and 2 ml bacterial suspension), an inoculum control (2 ml of sterile deionized water added to 2 ml bacterial suspension), and a cAMP (cyclic adenosine monophosphate, Sigma, St Louis, MO) control (1 ml of a 10 mg/ml filter-sterilized cAMP solution added to 1 ml of bacterial suspension). Cyclic adenosine monophosphate has been previously shown to partially inhibit tumor formation (3).

*Statistical analysis.* The experimental design was a randomized block. For each *Echinacea* source tested, one set was made of three replicates and three sub-replicates totaling 45 potato discs per fraction. Mean separations were determined using the Duncan's multiple range test. Experiments were repeated. Data from each experiment were analyzed separately, found to be similar statistically, and therefore pooled.

## RESULTS

Potato discs inoculated with ethanol fractions from Brands 2, 5 and 6 inhibited tumor induction between 41 and 59 percent as compared with controls without extract (Table 2). Acetone fractions derived from Brands 2 and 6 inhibited tumor formation by 39 and 46 percent, respectively. The water fraction obtained from Brand 5 suppressed tumor initiation by 38 percent compared with controls without extract. Analysis of variance of all *Echinacea* sources indicated a significant interaction of *Echinacea* source with solvent fraction. Application of the ethanol fraction of Brand 1 and the water fraction of Brand 7 resulted in 186 percent and 71 percent more tumors, respectively, as compared with controls receiving no *Echinacea* extract.

In general, DMSO did not significantly modify the induction of crown-gall tumors by *A. tumefaciens* (data not shown). Inhibition of tumor induction by cAMP varied with a maximum inhibition of 59 percent.

TABLE 2. The effect of *Echinacea* product extracts on formation of crown-gall tumors.

<i>Echinacea</i> supplement product <sup>1</sup>	Control water <sup>2</sup>	Control cAMP <sup>3</sup>	<i>Echinacea</i> extract		
			Acetone	Water	Ethanol
			(Number of tumors) <sup>4</sup>		
Brand 1	0.7b <sup>5</sup>	1.6ab	0.8b	1.5ab	2.0a
Brand 2	5.6a	3.3b	3.4b	nt <sup>6</sup>	2.3b
Brand 3	8.0ab	5.4b	6.5ab	4.9b	6.1ab
Brand 4	3.5ab	1.9b	2.4ab	nt	3.1ab
Brand 5	8.2a	6.3abc	5.9abc	5.1bc	4.7c
Brand 6	7.9a	3.2d	4.3cd	6.8ab	4.7bcd
Brand 7	3.4b	3.0b	5.1ab	5.8a	5.0ab
Brand 8	5.8a	4.1a	5.2a	4.9a	4.5a
Brand 9	2.4a	2.0a	1.7a	2.8a	1.7a

<sup>1</sup> All supplements were tested at maximum suggested daily dose.

<sup>2</sup> Sterile deionized water used in place of *Echinacea* extract.

<sup>3</sup> cAMP, compound with demonstrated antitumor activity (3), was used in place of *Echinacea* extract.

<sup>4</sup> Formed on potato tuber discs inoculated with *A. tumefaciens*.

<sup>5</sup> Mean separation within rows by Duncan's multiple range test,  $P = 0.05$ .

<sup>6</sup> Not tested.

## DISCUSSION

The ingredients (as reported by product labels) of the tested echinacea supplements varied widely. Only Brand 2 consisted exclusively of extracts of *Echinacea* roots. Brands 4, 5, and 7 contained aerial parts of *E. purpurea*, but no other *Echinacea* constituent. Brands 1 and 9, which had the same recommended maximum dose, had the same composition of aerial parts of *E. angustifolia* and *E. purpurea*, roots of *E. angustifolia*, and vitamin C. Brand 2, 3, 6, and 8 were standardized to 4 percent phenolic compounds. This compares with Wills and Stuart (30) observations that about 20 percent of retail *Echinacea* products tested in Australia had near-zero levels of caffeoyl phenols and alkylamides.

Gilroy et al. (16) compared the chemical composition with the ingredients listed on the container labels of 59 retail *Echinacea* products and found that 10 percent contained no measurable amounts of *Echinacea*, 48 percent did not contain the assayed species as labeled, and 57 percent of 21 standardized supplements did not contain the levels of compounds indicated. These reports raise the possibility that labels of the products



tested in our study did not accurately reflect their compositions, which could explain why similarly labeled supplements (such as Brands 1 and 9) did not yield similar results in the potato disc bioassay.

DMSO proved to be a good solvent to resuspend fractions because this solvent did not significantly interfere with the ability of *A. tumefaciens* to induce tumors. Cyclic AMP, which has been shown to exert tumor-inhibiting activity on animal cells (10), has been reported to reduce crown-gall tumor formation on "Red Russett" potato discs by 60 percent (12), a reduction similar to that measured in our study. Cyclic AMP inhibits tumor formation by increasing intercellular communication (11). In our experiment, the cAMP effect was variable and dependent on the bacterial culture. Since our experiment was statistically blocked on the *Echinacea* brand, variation in inhibition of tumor formation by cAMP could have resulted from variation inherent in the different potato tubers used for each brand. Differences inherent in the potato tubers and bacterial cultures likewise may have influenced the inhibitory effectiveness of each supplement.

Bioactivity of extractions could have been affected by post-harvest treatments of the *Echinacea* used in the products. Different types of processing, drying, and storage may have been used with the plant material and could explain differences the effects of the *Echinacea* on tumor formation (23). Levels of cichoric acid, a predominant phenolic in roots and aerial parts of *E. purpurea* (22) that may contribute to immunostimulatory activity (6), are known to decrease with increasing drying temperatures from 40°C to 70°C in roots and shoots of *E. purpurea* (25). Alkamides, major constituents of *E. purpurea* and *E. angustifolia* roots that demonstrate immunostimulatory activity (7), were degraded at 20°C and 30°C in *E. purpurea* when stored in light, but not when stored at 5°C in the dark (31).

Use of different extraction solvents results in different chemical composition in the solvent fractions. Alcoholic tinctures of *Echinacea* aerial parts and roots contain bioactive caffeoyl phenols and lipophilic, polyacetylene-derived compounds (5). Ethanol extracts of three supplements (Brand 2, 5, and 6) inhibited tumor formation by *A. tumefaciens*. Water extraction of ground aerial parts of *E. purpurea* or roots of *E. angustifolia* and *E. purpurea* resulted in over 50 percent loss of phenolic compounds, such as cichoric acid and caftaric acid, probably due to enzymatic browning mediated by polyphenol oxidase (22). The only suppressive water extract was derived from Brand 5. Since cichoric acid and caftaric acid are associated with immunostimulatory activity (6),



oxidation of phenolics by water extraction may explain why the water fraction of only one brand inhibited tumor formation.

Brands 1 and 9 of *Echinacea* products contained unspecified amounts of the bioflavonoid quercetin. In Chinese hamster lung fibroblast cells (V79), quercetin counteracted the inhibition of metabolic cooperation induced by chemical tumor promoters (28). Levels of quercetin and bioactive compounds from *Echinacea* in extracts of Brands 1 and 9, however, may have been too low to inhibit tumor formation. Although Brands 1 and 9 listed identical ingredients, fractions from Brand 9 had no effect on tumor formation and the ethanol fraction of Brand 1 increased the number of tumors compared with untreated controls. No fraction from Brand 4 suppressed tumor formation even though this product claimed to contain twice the amount of aerial parts of *E. purpurea* as Brand 5, which inhibited tumor development. Brand 7 had 40 percent more aerial parts of *E. purpurea* than Brand 5, but did not interfere with tumor formation. These results would be consistent with inaccurate product labeling or different harvest and handling practices that would degrade bioactive compounds.

In summary, crown-gall tumor formation was suppressed by acetone and ethanol extracts of Brands 2 and 6 and by water and ethanol extracts of Brand 5. The potato disc bioassay demonstrated that the labeled contents of the retail *Echinacea* products tested did not provide a basis for predicting the ability of the product to inhibit the development of crown-gall tumors.

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